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Biochemistry

## Human aspartic protease memapsin 2 cleaves the $\beta$ -secretase site of $\beta$ -amyloid precursor protein

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### ► Abstract

The cDNAs of two new human membrane-associated aspartic proteases, memapsin 1 and memapsin 2, have been cloned and sequenced. The deduced amino acid sequences show that each contains the typical *pre*, *pro*, and aspartic protease regions, but each also has a C-terminal extension of over 80 residues, which includes a single transmembrane domain and a C-terminal cytosolic domain. Memapsin 2 mRNA is abundant in human brain.

The protease domain of memapsin 2 cDNA was expressed in *Escherichia coli* and was purified. Recombinant memapsin 2 specifically hydrolyzed peptides derived from the  $\beta$ -secretase site of both the wild-type and Swedish mutant  $\beta$ -amyloid precursor protein (APP) with over 60-fold increase of catalytic efficiency for the latter. Expression of APP and memapsin 2 in HeLa cells showed that memapsin 2 cleaved the  $\beta$ -secretase site of APP intracellularly. These and other results suggest that memapsin 2 fits all of the criteria of  $\beta$ -secretase, which catalyzes the rate-limiting step of the *in vivo* production of the  $\beta$ -amyloid ( $A\beta$ ) peptide leading to the progression of Alzheimer's disease. Recombinant memapsin 2 also cleaved a peptide derived from the processing site of presenilin 1, albeit with poor kinetic efficiency. Alignment of cleavage site sequences of peptides indicates that the specificity of memapsin 2 resides mainly at the  $S_1'$  subsite, which prefers small side chains such as Ala, Ser, and Asp.

membrane aspartic proteases | Alzheimer's disease

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- ▲ [Top](#)
- [Abstract](#)
- ▼ [Introduction](#)
- ▼ [Materials and Methods](#)
- ▼ [Results](#)
- ▼ [Discussion](#)
- ▼ [References](#)

## ► Introduction

Many proteases are intimately involved in the regulation of cellular and physiological functions. There are five well studied human aspartic proteases. Pepsin and gastricsin participate in digestion in the stomach whereas cathepsins D and E function in intracellular protein degradation in lysosomes and endosomes. Human cathepsin D has been implicated in the metastasis of breast cancer and in Alzheimer's disease. Renin catalyzes the conversion of angiotensinogen to angiotensin 1, a clinically important step in hemostasis. The intimate involvement of human aspartic proteases in physiology and disease illustrates their central role in biology and medicine.

▲ <a href="#">Top</a>
▲ <a href="#">Abstract</a>
• <a href="#">Introduction</a>
▼ <a href="#">Materials and Methods</a>
▼ <a href="#">Results</a>
▼ <a href="#">Discussion</a>
▼ <a href="#">References</a>

The emergence of human gene sequences in the expressed sequence tag (EST) database represents an important new resource for identifying novel human enzymes. We report here the cloning of two new human aspartic proteases, memapsin 1 (M1) and memapsin 2 (M2), initially identified from the human EST database. These proteases are unique among aspartic proteases in that they are membrane-anchored. The presence of M2 in the brain led us to test its capacity to hydrolyze the  $\beta$ -amyloid precursor protein (APP). Detailed enzymic and cellular studies suggest that M2 fits all of the criteria of  $\beta$ -secretase. Like M2, APP is a type I integral transmembrane protein and is known to be processed *in vivo* at three sites. The evidence suggests that cleavage at the  $\alpha$ -secretase site by a membrane-associated metalloprotease is a physiological event. This site is located in APP 12 residues away from the luminal surface of the plasma membrane. Cleavage of the  $\beta$ -secretase site (28 residues from the plasma membrane's luminal surface) and the  $\gamma$ -secretase site (in the transmembrane region) results in a 40/42-residue  $\beta$ -amyloid peptide ( $A\beta$ ), whose elevated production and accumulation in the brain are the central events in the pathogenesis of Alzheimer's disease (for review, see ref. 1). Presenilin 1, another membrane protein found in human brain, controls the hydrolysis at the APP  $\gamma$ -secretase site and has been postulated to be itself the responsible protease (2). Presenilin 1 is expressed as a single chain molecule, and its processing by a protease, presenilinase, is required to prevent it from rapid degradation (3, 4). The identity of presenilinase is unknown. The *in vivo* processing of the  $\beta$ -secretase site is thought to be the rate-limiting step in  $A\beta$  production (5) and, thus, is a strong therapeutic target. The production of recombinant M2 in *Escherichia coli* and the information on its specificity reported here should be useful for the design and testing of potential inhibitor drugs.

## ► Materials and Methods

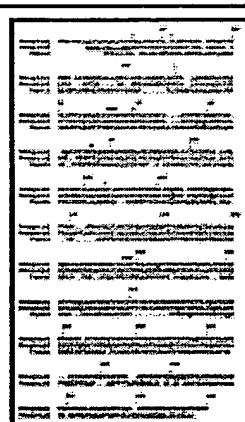
**Cloning of Memapsin 2.** Three new EST sequences homologous to human aspartic proteases were found in EST database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) (EST [AA136368](#), from human pregnant uterus; EST [AA207232](#), from human neurepithelium; EST [R55398](#), from human breast) using human aspartic protease motifs as a query. The corresponding bacterial strains 947471, 214526, and 392689 containing the EST sequences were obtained from the American Type Culture Collection. The completed sequences from these clones assembled

▲ <a href="#">Top</a>
▲ <a href="#">Abstract</a>
▲ <a href="#">Introduction</a>
• <a href="#">Materials and Methods</a>
▼ <a href="#">Results</a>
▼ <a href="#">Discussion</a>
▼ <a href="#">References</a>

into  $\approx 80\%$  of *prepro*-M2 cDNA. The 5' region of M2 cDNA was obtained by using 5' rapid amplification of cDNA ends PCR, which was carried out by using human pancreas Marathon-Ready cDNA (CLONTECH) and human pancreatic  $\lambda$ -gt10 and  $\lambda$ -gt11 libraries as templates and M2 specific primers. *Prepro*-M1 cDNA was cloned by using a similar procedure.

**Tissue Distribution of Memapsin 1 and 2 mRNA.** Multiple human tissue cDNA panels (CLONTECH) were used as template for PCR amplification (30 cycles) of a 0.56-kb memapsin 1 cDNA fragment using primers M1F1 (5'-GACGTCACATGTAAG-TACACACAAGGAAGC) and M1R2 (5'-CAGAGATGCGCGGGCCACAGCTTCCACCAC), and of a 0.82-kb memapsin 2 cDNA fragment using primers M2F1 (5'-GGTAAGCATCCCCCATGGCCCCAACGTC-3') and M2R2 (5'-CCAATTCGTTTTTCGGGCCCCGATCAAAGACAACG-3'). The amplified fragments were compared in agarose gel electrophoresis.

***E. coli* Expression of the Protease Domain of *pro*-Memapsin 2.** A fragment of M2 cDNA encoding the estimated *pro* and protease domain (without the C-terminal extension) were PCR amplified and inserted into the *Bam*HI site of vector pET11a. The resulting vector, pET11-PM2<sub>pd</sub>, expresses a protein, *pro*-M2<sub>pd</sub>, whose sequence starts from Ala<sup>-8P</sup> to Ala<sup>326</sup> [pepsin residue numbering (Fig. 1)]. The expression and purification of M2<sub>pd</sub> was carried out in *E. coli* strain BL21(DE3) as previously described (6) with minor modifications as follows: inclusion bodies dissolved in 8 M urea were rapidly diluted into 20 mM Tris base, and the pH was adjusted to 8.0.



**Fig. 1.** Predicted amino acid sequence of human *prepro*-memapsin 1 (GenBank accession no. AF200192) and *prepro*-memapsin 2 (GenBank accession no. AF200193) aligned against the sequence of human *pre*-pepsinogen. Amino acid codes are according to standard International Union of Pure and Applied Chemistry nomenclature. The beginning of *pro* and mature protease regions of pepsinogen are marked by residue numbers 1P and 1, respectively. Two active-site aspartic acids in D(T/S)G motifs are marked by ■ and conserved Tyr<sup>75</sup> by ♦. Residues in the predicted transmembrane domains are in boldface.

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**Activation of *pro*-Memapsin 2 and Enzyme Kinetics.** Incubation in 0.1 M sodium acetate (pH 4.0) for 16 h at 22°C autocatalytically converted *pro*-M2<sub>pd</sub> to M2<sub>pd</sub>. For initial hydrolysis tests, two synthetic peptides were separately incubated with *pro*-M2<sub>pd</sub> in 0.1 M Na acetate (pH 4.0) for different periods ranging from 2 to 18 h. The incubated samples were subjected to HPLC/mass spectroscopy for the identification of the hydrolytic products (average error in mass determination was 0.02%). For kinetic studies, the identified HPLC (Beckman System Gold) product peaks were

integrated for quantitation. The  $K_m$  and  $k_{cat}$  values for presenilin 1 and Swedish-APP peptides (Table 1) were measured by steady-state kinetics. The individual  $K_m$  and  $k_{cat}$  values for APP peptide could not be measured accurately by standard methods, so its  $k_{cat}/K_m$  value was measured by competitive hydrolysis of mixed substrates against presenilin 1 peptide (7).

**View this table:** **Table 1.** Alignment on amino acids appearing in eight subsites of memapsin substrates  
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**Mammalian Cell Transfections.** *Pro*-M2 cDNA was cloned into the *EcoRV* site of vector pSecTag A (Invitrogen). Human APP cDNA was PCR amplified from human placenta  $\lambda$ -gt11 library (CLONTECH) and was cloned into the *NheI* and *XbaI* sites of pSecTag A. The procedure for transfection into HeLa cells and vaccinia virus infection for T7-based expression are essentially the same as described (8).

**Pulse-Chase and Immunodetection.** Transfected cells were metabolically labeled with 200  $\mu$ Ci of  $^{35}$ S methionine and cysteine (TransLabel; ICN) in 0.5 ml of serum-free/methionine-free media for 30 min, were rinsed with 1 ml of media, and were replaced with 2 ml of DMEM/10% FCS. To block vesicle acidification, bafilomycin A1 was included in the media (9). At different time points (chase), media was removed and the cells were harvested and lysed in 50 mM Tris, 0.3 M NaCl, 5 mM EDTA, and 1% Triton X-100 (pH 7.4) containing 10 mM iodoacetamide, 10  $\mu$ M TPCK, 10  $\mu$ M TLCK, and 2  $\mu$ g/ml leupeptin. The supernatant ( $14,000 \times g$ ) of cell lysates and media were immunoabsorbed onto antibody bound to protein G Sepharose (Sigma). Anti-APP N-terminal domain antibody (Chemicon) was used to recover the N $\beta$ -fragment of APP, and anti-A $\beta_{1-17}$  antibody (Chemicon, recognizing the N-terminal 17 residues of A $\beta$ ) was used to recover the 12 kDa  $\beta$ C-fragment. The former antibody recognized only denatured protein, so media was first incubated in 2 mM dithiothreitol and 0.1% SDS at 55°C for 30 min before immunoabsorption. Samples were cooled and diluted with an equal volume of cell lysis buffer before addition of anti-APP N-terminal domain (Chemicon). Beads were washed, were eluted with loading buffer, were subjected to SDS/PAGE (NOVEX, San Diego), and were visualized by autoradiogram or PhosphorImaging (Molecular Dynamics) on gels enhanced with Amplify (Amersham). Immunodetection of the N $\alpha$  fragment was accomplished by transblotting onto a PVDF membrane and detecting with anti-A $\beta_{1-17}$  and chemiluminescent substrate (Amersham).

**Cellular Localization of M2 and APP.** HeLa cells transfected with APP or M2 (see above) in four-well chamber slides were fixed with acetone for 10 min and were permeabilized in 0.2% Triton X-100 in PBS for 6 min. For localizing M2, polyclonal goat anti-*pro*-M2<sub>pd</sub> antibodies were purified on DEAE-Sepharose 6B and were affinity-purified against recombinant *pro*-M2<sub>pd</sub> immobilized on Affigel (Bio-Rad). Purified anti-*pro*-M2<sub>pd</sub> antibodies were conjugated to Alexa568 (Molecular Probes) according to the manufacturer's protocol. Fixed cells were incubated overnight with a 1:100

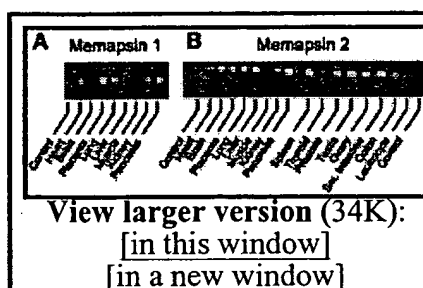
dilution of antibody in PBS containing 0.1% BSA and were washed four times with PBS. For APP, two antibodies were used: antibody A $\beta_{1-17}$  (described above) and antibody A $\beta_{17-42}$ , which recognizes the first 26 residues after the  $\alpha$ -secretase cleavage site (Chemicon). After four PBS washes, the cells were incubated overnight with an anti-mouse FITC conjugate at a dilution of 1:200. Cells were mounted in Prolong anti-fade reagent (Molecular Probes) and were visualized on a Leica (Deerfield, IL) TCS confocal laser scanning microscope.

## ► Results

EST clones from three human cDNAs were identified from homology search based on aspartic protease motifs and were sequenced after 5' rapid amplification of cDNA ends. The deduced protein sequences (Fig. 1) are characteristic of mammalian aspartic protease zymogens. Uniquely, each enzyme also has a C-terminal extension of over 80 residues (see Fig. 1 alignment with pepsin), which includes a putative transmembrane region.

The enzymes are named memapsin 1 and memapsin 2 (for membrane-anchored aspartic protease of the pepsin family; GenBank accession nos. [AF200192](#) and [AF200193](#), respectively). In human tissues, spleen and prostate have the highest levels of M1 mRNA level [but is absent in the brain (Fig. 2A)] whereas pancreas and brain have the highest amount of M2 (Fig. 2B). To search for M2 substrates among membrane proteins of the brain, the estimated *pro*-M2 protease domain (*pro*-M2<sub>pd</sub>, without the C-terminal extension) was expressed in *E. coli* as inclusion bodies, refolded and purified. In acidic solutions, *pro*-M2<sub>pd</sub> spontaneously generated activity (see below) with the concomitant appearance of smaller fragments (Fig. 1, Fig. 3A, and Table 1), a common property for aspartic protease zymogens. Whether this activity is produced by the classical intramolecular activation of aspartic protease zymogens (10, 11) is presently unknown. For the current discussion, we will refer to this activity as that of M2 (M2<sub>pd</sub>).

- ▲ [Top](#)
- ▲ [Abstract](#)
- ▲ [Introduction](#)
- ▲ [Materials and Methods](#)
- [Results](#)
- ▼ [Discussion](#)
- ▼ [References](#)



**Fig. 2.** Tissue distribution of M1 (A) and M2 (B) mRNA determined by reverse transcription-PCR visualized in agarose gel electrophoresis. Control experiment contained no template DNA. Amplification of glyceraldehyde-3-phosphate dehydrogenase mRNA produced uniform intensity (data not shown).

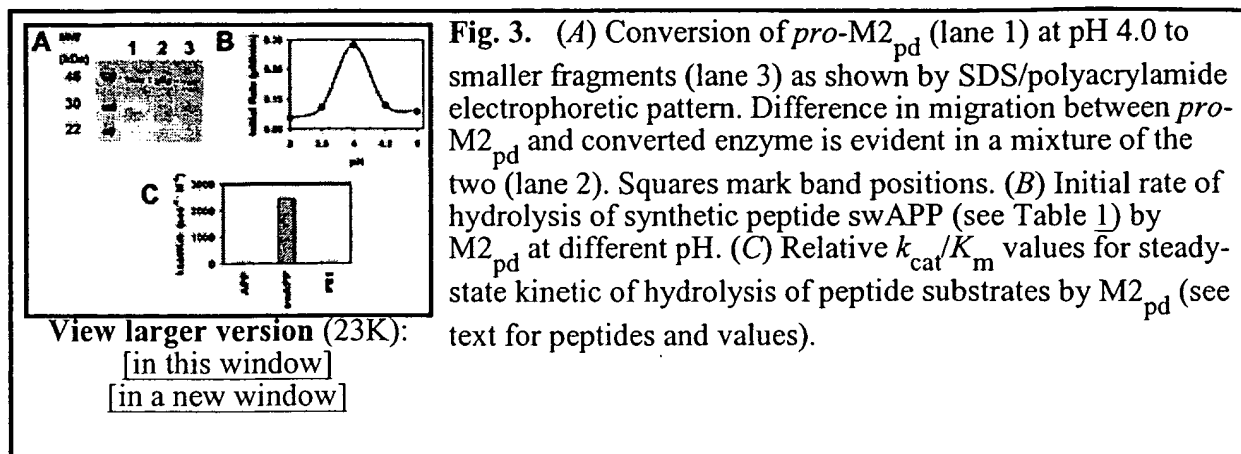
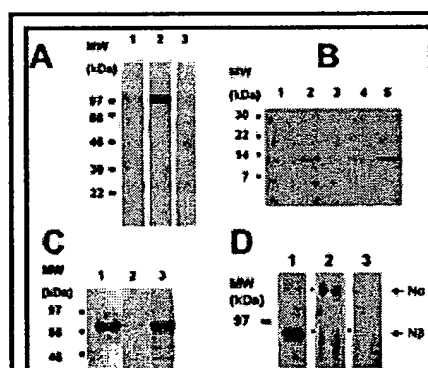


Fig. 3. (A) Conversion of *pro*-M2<sub>pd</sub> (lane 1) at pH 4.0 to smaller fragments (lane 3) as shown by SDS/polyacrylamide electrophoretic pattern. Difference in migration between *pro*-M2<sub>pd</sub> and converted enzyme is evident in a mixture of the two (lane 2). Squares mark band positions. (B) Initial rate of hydrolysis of synthetic peptide swAPP (see Table 1) by M2<sub>pd</sub> at different pH. (C) Relative  $k_{cat}/K_m$  values for steady-state kinetic of hydrolysis of peptide substrates by M2<sub>pd</sub> (see text for peptides and values).

Modeling of M2 three-dimensional structure as a type I integral membrane protein suggested that its globular protease unit can hydrolyze a membrane-anchored polypeptide at a distance range of 20-30 residues from the membrane surface (results not shown). As a transmembrane protein of the brain, APP is a potential substrate. Its  $\beta$ -secretase site, located about 28 residues from the plasma membrane surface, is within in the range for M2 proteolysis. A synthetic peptide derived from this site (SEVKM/DAEFR) was hydrolyzed by M2<sub>pd</sub> at the  $\beta$ -secretase site (marked by a slash): A second peptide (SEVNL/DAEFR) derived from APP  $\beta$ -secretase site with the "Swedish mutation" (12), known to elevate the level of A $\beta$  production in cells (13), was hydrolyzed by M2<sub>pd</sub> with much higher catalytic efficiency (Fig. 3C). Both substrates were optimally cleaved at pH 4.0 (Fig. 3B). A peptide derived from the processing site of presenilin 1 (SVNM/AEGD) was also cleaved by M2<sub>pd</sub> with poor kinetic parameters (Fig. 3C). A peptide derived from the APP  $\gamma$ -secretase site (KGGVVIATVIVK) was not cleaved by M2<sub>pd</sub>. Interestingly, pepstatin A inhibited M2<sub>pd</sub> poorly ( $IC_{50} \approx 0.3$  mM). The kinetic parameters indicate that both presenilin 1 ( $k_{cat}$ ,  $0.67$  s<sup>-1</sup>;  $K_m$ ,  $15.2$  mM;  $k_{cat}/K_m$ ,  $43.8$  s<sup>-1</sup>·M<sup>-1</sup>) and native APP peptides ( $k_{cat}/K_m$ ,  $39.9$  s<sup>-1</sup>·M<sup>-1</sup>) are very poor substrates whereas Swedish APP peptide ( $k_{cat}$ ,  $2.45$  s<sup>-1</sup>;  $K_m$ ,  $1$  mM;  $k_{cat}/K_m$ ,  $2,450$  s<sup>-1</sup>·M<sup>-1</sup>) is a much better substrate.

To determine whether M2 possesses an APP  $\beta$ -secretase function in mammalian cells, these two cDNAs were transiently expressed in HeLa cells (8), were metabolically pulse-labeled with <sup>35</sup>S-Met, and then were immunoprecipitated with anti-APP antibodies for visualization of APP-generated fragments after SDS/polyacrylamide electrophoresis and imaging. Cells expressing both APP and M2 produced the 97-kDa APP N $\beta$ -fragment (from the N terminus to the  $\beta$ -secretase site) in the conditioned media (Fig. 4A, lane 2) and the 12-kDa  $\beta$ C-fragment (from the  $\beta$ -secretase site to the C terminus) in the cell lysate (Fig. 4B, lane 2). Controls that included transfection of APP alone produced little detectable N $\beta$ -fragment (Fig. 4A, lane 1) nor the  $\beta$ C-fragment (Fig. 4B, lane 1). Bafilomycin A1, which is known to raise the intravesicle pH of lysosomes/endosomes and has been shown to inhibit APP cleavage by  $\beta$ -secretase (14), abolished the production of both APP fragments N $\beta$  and  $\beta$ C (Fig. 4A, lane 3 and Fig. 4B, lane 3, respectively) in co-transfected cells. Transfection of cells with Swedish APP alone did not produce the  $\beta$ C-fragment band in the cell lysate (Fig. 4B, lane 4), but the co-transfection of Swedish APP and M2 did (Fig. 4B, lane 5). This Swedish  $\beta$ C-fragment band is more intense than that of the wild-type APP (Fig. 4B, lane 2). A 97-kDa N $\beta$ -band is also seen

in the conditioned media but is about the equal intensity as the wild-type APP transfection (result not shown). These results suggest that M2 processes the  $\beta$ -secretase site of APP in acidic compartments such as the endosomes. To establish the expression of transfected M2 gene, the pulse-labeled cells were lysed and immunoprecipitated by anti-M2 antibodies. A 70-kDa M2 band was seen in cells transfected with M2 gene (Fig. 4C, lane 1), which has the same mobility as the major band from HEK 293 cells (Fig. 4C, lane 3) known to express  $\beta$ -secretase (13). A very faint band of M2 is also seen, after a long film exposure, in untransfected HeLa cells (Fig. 4C, lane 2), indicating a very low level of endogenous M2, which was insufficient to produce  $N\beta$ - or  $\beta$ C-fragments without M2 transfection (Fig. 4A, lane 1 and Fig. 4B, lane 1). Antibody  $A\beta_{1-17}$ , which specifically recognizes residues 1-17 in  $A\beta$  peptide, was used to confirm the correct  $\beta$ -secretase site cleavage. From immunoprecipitation of conditioned media from cells transfected with APP and M2, both  $N\alpha$ - and  $N\beta$ -fragments are visible using an antibody recognizing the N-terminal region of APP present in both fragments (Fig. 4C, lane 1). In Western blots, antibody  $A\beta_{1-17}$  recognized the immunoprecipitated  $N\alpha$ -fragment produced by endogenous  $\alpha$ -secretase in the untransfected cells (Fig. 4C, lane 2). This antibody was, however, unable to recognize the  $N\beta$ -fragment (Fig. 4C, lane 3) known to be present in cells co-transfected with APP and M2 (compare the 95-kDa band in lanes 1 and 3). These observations confirmed that  $N\beta$ -fragment is the product of  $\beta$ -secretase site cut by M2, which abolished recognition of the epitope of  $A\beta_{1-17}$ .



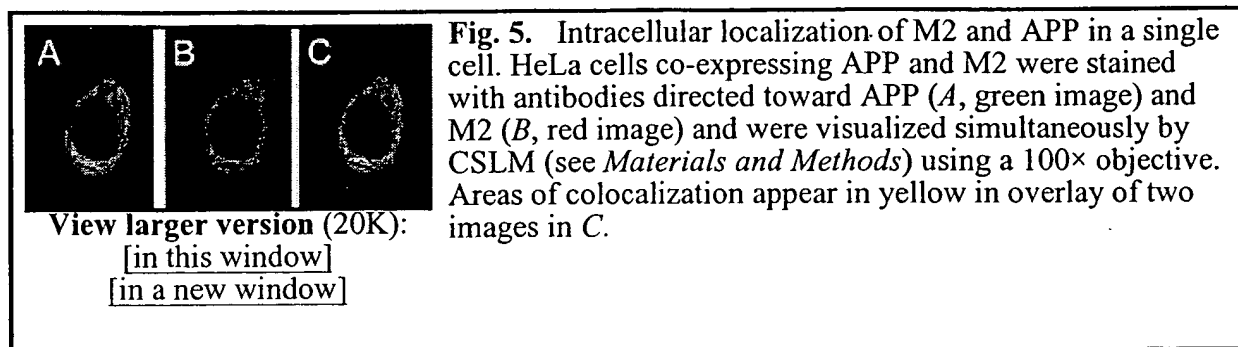
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**Fig. 4.** Processing of APP by M2 in HeLa cells. (A) SDS/PAGE patterns of immunoprecipitated APP  $N\beta$ -fragment (97-kDa band) from the condition media (2 h) of pulse-chase experiments. Lanes: 1, transfection of APP alone; 2, co-transfection of APP and M2; 3, same as lane 2 except that bafilomycin A1 is included. (B) SDS/PAGE patterns of APP  $\beta$ C-fragment (12 kDa) immunoprecipitated from the conditioned media of the same experiment as in A. Lanes: 1, transfection of APP; 2, co-transfection of APP and M2; 3, as in lane 2 but with bafilomycin A1; 4, transfection of Swedish APP; 5, co-transfection of Swedish APP and M2. (C) SDS/PAGE patterns of immunoprecipitated M2 (70 kDa). Lanes: 1, M2 transfected cells; 2, untransfected HeLa cells after long time film exposure; 3, endogenous M2 from HEK 293 cells. (D) SDS/PAGE patterns of APP fragments (100-kDa  $N\alpha$ -fragment and 95-kDa  $N\beta$ -fragment) recovered from conditioned media after immunoprecipitation using antibodies specific for the N-terminal region of APP. Lanes: 1, autoradiogram of immunoprecipitation from co-transfection of APP and M2; 2 and 3, immunoblotted by using antibody  $A\beta_{1-17}$ , specific for the  $N\alpha$ -fragment that does not recognize the  $N\beta$ -fragment; 2, transfection with APP alone; 3, co-transfection of APP and M2. Dots mark band positions.

The processing of APP by M2 predicts the intracellular colocalization of the two proteins. Immunodetection observed by confocal microscopy of both APP (Fig. 5A) and M2 (Fig. 5B) revealed



their colocalization in the superimposed scans (Fig. 5C). The distribution of both proteins is consistent with their residence in lysosomal/endosomal compartments.



In specificity studies, we found that M2<sub>pd</sub> cleaved its *pro* peptide (two sites) and the protease portion (two sites) during a 16-h incubation after activation (Table 1). Besides the three peptides discussed above, M2<sub>pd</sub> also cleaved oxidized bovine insulin B chain and a synthetic peptide Nch. Interestingly, we found that native proteins were not cleaved by M2<sub>pd</sub> (including  $\beta$ -amylase, alcohol dehydrogenase, carbonic anhydrase, cytochrome C, bovine and human serum albumin, hemoglobin, and chicken egg lysozyme; data not shown). The residues around all of these cleavage sites are aligned in Table 1. The specificity of M2 derived from these results will be further discussed below.

## ► Discussion

The data presented herein strongly suggest that human M2 fulfills all of the criteria of a  $\beta$ -secretase that cleaves the  $\beta$ -amyloid precursor protein (APP):

(i) M2 and APP are both membrane proteins present in human brain and co-localize in mammalian cells, (ii) M2 specifically cleaves the  $\beta$ -secretase site of synthetic peptides and of APP in cells, (iii) M2 preferentially cleaves the  $\beta$ -secretase site from the Swedish over the wild-type APP, and (iv) the acidic pH optimum for M2 activity and bafilomycin A1 inhibition of APP processing by M2 in the cells are consistent with the previous observations that  $\beta$ -secretase cleavage occurs in acidic vesicles (14). The spontaneous appearance of activity of recombinant pro-M2 in an acidic solutions is consistent with the idea that this zymogen can by itself generate activity in an acidic vesicle like an endosome. However, we note that the cleavage sites (Table 1) are not near the vicinity of the aligned pepsin N terminus (Fig. 1). It seems probable that the activation of proM2 *in vivo* may require another protease. While preparing this manuscript, the cloning of a human transmembrane aspartic protease BACE and its activity in the cleavage of APP  $\beta$ -secretase site were reported (15). The sequences of BACE and *pro*-memapsin 2 are identical. Although the approaches used for cloning, transfection, and expression were different in these two studies, both studies concluded that the new protease is  $\beta$ -secretase, the long-sought enzyme inexorably involved in Alzheimer's disease.

▲ <a href="#">Top</a>
▲ <a href="#">Abstract</a>
▲ <a href="#">Introduction</a>
▲ <a href="#">Materials and Methods</a>
▲ <a href="#">Results</a>
• <a href="#">Discussion</a>
▼ <a href="#">References</a>

We have determined that the hydrolysis of the Swedish peptide by M2<sub>pd</sub> has a  $k_{cat}/K_m$  value  $\approx 60\times$

over that for the wild-type APP peptide, which explains the higher production of A $\beta$  peptide and the early onset of Swedish familial Alzheimer's disease (12). The relative amount of  $\beta$ -secretase cleavage of APP and Swedish APP estimated from the cellular experiments is only about six-fold in favor of Swedish APP (13). We also observed only a few-fold increase of the  $\beta$ C-fragment from Swedish APP over that of native APP (Fig. 4B). Although  $\beta$ -secretase is known to be the rate-limiting step in the production of A $\beta$  peptide from native APP (5), the cellular processing of Swedish APP, with 60 $\times$  of catalytic efficiency, would likely make other steps in A $\beta$  production to be rate limiting. The correct cleavage of a peptide from the processing site of presenilin 1 by M2<sub>pd</sub> (Table 1) could be taken to suggest that M2 may be the presenilin 1 processing protease, presenilinase. This seems to be improbable because the kinetics are not sufficiently robust for the required physiological efficiency. There may also be a question of accessibility of the presenilinase site by M2 that is located on the luminal side of the membrane. Evidence for the localization of the processing loop of presenilin 1 on the luminal (16) and cytosolic (17) sides of the membrane have both been reported.

Data presented in Table 1 suggest that M2 has an overall broad substrate specificity. Among the eight subsites, present in all aspartic proteases, the most stringent specificity for M2 occurs at S<sub>1</sub>', which prefers small or negatively charged side chains, such as Ala, Asp, and Ser, or no side chain, Gly. Our data on the P<sub>1</sub> preference are in general agreement but somewhat broader than the results of a cellular mutagenesis study (18) in which Tyr, Leu, Phe, and Met were found at P<sub>1</sub>. This is expected from a higher sensitivity for product detection in our experiments. Other subsites show relatively high tolerance for side chains of different sizes and charges. From the sequences of the peptide substrates that were cleaved, there appears to be a trend of requiring at least two large hydrophobic residues in these eight subsites. These specificity findings suggest that the P<sub>1</sub>/P<sub>1</sub>' residues at the  $\alpha$ -secretase site (Lys/Leu) and the  $\gamma$ -secretase site (Val/Ile and Ala/Thr) are not favored by M2 and would predict that they are not substrates. The failure of M2 to cleave native proteins is consistent with the requirement in a substrate of a stretch of peptide in an extended conformation. The hydrolysis of Nch peptide between residues 1-2 and 3-4 (Table 1) suggests that the hydrolysis by M2 does not require a substrate to fill all eight substrate binding subsites. This may indicate that a relatively short peptidic transition-state analogue can be a good inhibitor for M2.

Even though the molecular architecture of the two memapsins are similar, because M1 is absent in the brain (Fig. 2A), the question as to whether it can cleave the APP  $\beta$ -secretase site may be physiologically irrelevant. However, the presence of both M1 and M2 in many other tissues argues for important roles of this new class of aspartic proteases in other physiological processes. The poor kinetic parameters for the hydrolysis of  $\beta$ -secretase site of native APP by M2 suggest that this cleavage may be a tolerated aberration in normal human brain.

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## ► Abbreviations

APP,  $\beta$ -amyloid precursor protein; M1, memapsin 1; M2, memapsin 2; EST, expressed sequence tag.

## ► Footnotes

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. [AF200192](#) (memapsin 1) and [AF200193](#) (memapsin 2)].

## ► References

▲ <a href="#">Top</a>
▲ <a href="#">Abstract</a>
▲ <a href="#">Introduction</a>
▲ <a href="#">Materials and Methods</a>
▲ <a href="#">Results</a>
▲ <a href="#">Discussion</a>
• <a href="#">References</a>

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